Biochemical Problems in Determining the Age of Bruised Animal Tissue

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The Farmer Cooperative Service conducts research studies and service activities of assistance to farmers in connection with cooperatives engaged in marketing farm products, purchasing farm supplies, and supplying business services. The work of the Service relates to problems of management, organization, policies, merchandising, product quality, costs, efficiency, and membership.

The Service publishes the results of the studies; confers and advises with officials of farmer cooperatives; and works with educational agencies, cooperatives, and others in the dissemination of information relating to cooperative principles and practices.

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This study was conducted under authority of the Agricultural Marketing Act of 1946 (RMA, Title II).

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BIOCHEMICAL PROBLEMS IN DETERMINING THE AGE OF BRUISED ANIMAL TISSUE

The losses due to damaged livestock prior to slaughter account for many millions of dollars each year. Some of these losses are readily apparent---animals which arrive at market or packing plant crippled or dead. Other losses may not be visible prior to actual slaughter---occurring in animals which are condemned in whole or in part because of disease, parasitic ravages, or bruising. If these visible and hidden losses are to be reduced, there must be an awareness of the damage problem, concern for its reduction and control, and a program for alleviation through actions on the part of producers, transporters, marketers, and all others who have a stake in the livestock and meat industry.

In seeking methods to reduce losses, one of the greatest problems is the fixing of responsibility for the particular loss. This problem is particularly acute in the instance of bruise loss. Since animals can be bruised at any time up to slaughter, it is impossible to assess the responsibility for bruise damage unless the occasion of injury is so evident as to be positively known. The most logical approach to a solution of this problem appeared to be through a determination of the age of the bruised tissue. If the age of the bruise can be determined, the time of injury can be ascertained and by associating the whereabouts of the animal with the time established, a positive means of affixing responsibility can be provided.

This report is based on work done by the Ohio Agricultural Experiment Station and the Ohio State University under a contract with Farmer Cooperative Service, with funds provided under the Agricultural Marketing Act. The project

has been conducted by Farmer Cooperative Service as a means of assisting producers in reducing livestock marketing losses.

This report has been prepared by Joseph E. Rickenbacker, Farmer Cooperative Service, on the basis of reports submitted by the contractor and on the following technical papers:

Hamdy, M. K., Deatherage, F. E., and Shinowara, G. Y., Bruised Tissue. I. Biochemical Changes Resulting From Blunt Injury., Proceedings of the Society for Experimental Biology and Medicine, 1957, v95, 255-258.

Hamdy, M. K., Kunkle, L. E., and Deatherage, F. E., Bruised Tissue. II. Determination of the Age of a Bruise., Journal of Animal Science, Vol. 16, No. 2, May 1957.

Hamdy, M. K., Kunkle, L. E., Rheins, M. S., and Deatherage, F. E., Bruised Tissue. III. Some Factors Affecting Experimental Bruises., Journal of Animal Science, Vol. 16, No. 2, May 1957.

The work at Ohio was under the direction of Dr. F. E. Deatherage, Chairman, Department of Agricultural Biochemistry, with the assistance of members of the staffs of the Departments of Animal Science, Pathology, and Agricultural Biochemistry of the University and the Experiment Station.

Objectives of the Study

The basic objective of this study was to provide a means of solving the problem of determining the time of bruising. If such a means could be evolved, then responsibility could be assessed and effective remedial measures undertaken. It was believed that the most practical way of arriving at "time of bruise"

would be to determine the "age of the bruise" by means of a simple test applied to the bruised tissue. This approach is directly related to the process whereby wounds are healed since a precise knowledge of the sequential processes which damaged tissue undergo during repair would indicate the age of the bruise and hence the time of bruising.

Finding a test which would definitely fix time of bruising required an intensive and fundamental study on the subtle aspects of wound healing. In order to find a suitable test it was necessary that at least one facet of the healing process be found which was independent of all factors except that of time. Thinking of this research program in these terms, it was possible to have a base upon which to project detailed investigations. From this point of view the main purposes of this investigation were studied. Over-all objectives of the project were:

(1) A study of the factors contributing to wounds and wound healing in most livestock;

(2) A study of the damaged tissue to determine a suitable test to estimate the time elapsed between bruising and examination of tissue.

Morphological Changes in Bruised Tissue

The importance of wound healing as an approach to the problem of age determination of a bruise led to studies of bruised tissue under microscopic examination. These studies appeared to confirm a definite sequence of events relative to the repair of damaged tissue (Table 1). These may be described as follows:

Diffuse, massive or linear hemorrhage into the muscle bundles as well as the connective and adipose tissues with considerable amounts of plasma and intact erythrocytes was seen in bruises of less than 1 hour's standing. The inflammatory process was not very evident as most of the leukocytes seen apparently originated from extravasated blood. Hypertrophy of the endothelial lining was seen, while the plasma accumulations seemed slightly fibrinoid in appearance. Some hyalinization of the sarcoplasm was occasionally observed, individual myofibrils became indistinct and some cross-striations might have been lost. Some fragmentation of the muscle elements was also present and occasionally there was a separation of the myofibrils.

Between 1 and 6 hours most of these changes persisted and some even progressed:—partial lysis of the sarcoplasm in certain fibers was seen although cross-striations in some of the same fibers was evident. Definite laying-down of fibrin was seen and some alterations in the red blood cells were noted--particularly swelling and a distortion of their normal biconcave shape. The inflammatory process appeared to be well underway especially in the peripheral regions of the wound.

Up to 10 hours a further crenation and angularity in the shape of erythrocytes was observed and numerous neutrophils could be seen in the exudate. A large number of these polymorphonuclear leukocytes were unsegmented.

At about 1 day's duration lymphocytes and monocytes as well as neutrophils were infiltrating among the muscle bundles while erythrocytes showed some hyalinization. Active erythrophagia was present. The band and segmented neutrophils and monocytes in the cellular exudate increased at the expense

Table 1. - Progression of gross changes occurring in a bruised tissue

Age of bruise	Gross observations on the live animal	Observations upon sectioning the bruise
Zero - 1 hour	Inflammation without laceration, diffused swelling, edema and the bruised area acquired a dark red color.	Acute hemorrhage and blood seeping out of the lesion were noted. The bruised tissues are dark red in color.
1 - 5 hours	The intensity of the dark red color increased and changed to purple-violet color.	
24 - 48 hours	The inflammation and the swelling subsided. The purple-violet color was still present.	The tissues looked reddish-brown in color. Sometimes small soft blood clots were observed.
2 - 3 days	Green color was detected at the periphery of the bruise which was still purple-violet in color.	Yellow fluid mixed with brown material was noticed seeping out of the lesion. Degenerated tissues were noticed mixed with blood.
3 - 4 days	Yellow color mixed with green (orange) color was observed at the periphery of the bruise while the purple color decreased in intensity.	Blood and yellow fluid were still present. No clots were observed. Degenerated tissue was noticed.
4 - 5 days	Peak of the orange color coinciding with the decrease of the purple color.	Yellow fluid was still present. The lesions were dark brown in color.
6 - 7 days	Decrease of orange color intensity and the disappearance of the purple color.	No fluid. The tissue seemed to be covered with yellow color while the inside was slightly brown.
7 - 10 days	The bruised area appeared morphologically normal.	The tissue showed normal appearance.

of erythrocytes and some phagocytes invaded the connective tissue stroma. Muscle bundles adjoining the areas of exudation showed a patchy distribution of hypochromic areas within which cross-striations were either partially or completely lost. At times the sarcoplasm was contiguous between adjacent fibers.

A bruise 2 days old showed marked anisocytosis of its extravasated red blood cells, many of which were in an advanced stage of haemolysis, assuming the appearance of "ghost cells". Polymorphonuclear neutrophils were sometimes scarcer in the exudate but the odd plasma cells were occasionally seen. Some of the erythrocytes conglutinated within the muscle fibres liberated their hemoglobin content and some diffuse staining of the tissue or deposition of pigmented plaque-like masses took place. The network of fibrin was becoming organized. Wherever the continuity of muscle was interrupted, fibroblastic proliferation from the perimysium was seen--an attempt of repair by substitution --and nuclei of the endomysium were also actively proliferating. Fibroblasts also appeared in the hemorrhagic areas of the adipose and connective tissue in swine. At times the sarcoplasm exhibited a lytic vacuolization (hydropic degeneration) and many muscle fibers began to atrophy or disappear entirely with only the sarcolemma remaining to outline their former presence. This membrane then enveloped an agglomeration of amorphous, granular, pale, eosinophillic cellular debris. The extensive cellular reaction was doubtlessly a response to the presence of this great amount of dying and dead tissue.

At 3 to 5 days the exudate became even more cellular with further proliferation of monocytes.

On the sixth and succeeding days much necrosis and possibly mineralization of the dead muscle fibers were seen, while at the same time myoblasts began to make their appearance in abortive attempts at repair by regeneration. Monocytic giant cells were evident in areas surrounding the dead tissue and the proliferation of fibroblasts from the endomysium was even more marked at this stage. Neocapillaries were being formed to accompany the fibrosis and most connective tissue fibers newly formed were of the reticular rather than the collagenous type.

Biochemical Changes in Bruised Tissue

In addition to microscopic examination of bruised tissue, investigations of the biochemical changes occurring in such tissue were undertaken. Cattle, sheep, and hogs were used in these studies.

The tissues were examined grossly and chemically immediately after bleeding and slaughter, excised and sliced for further testing.

Some analyses used. For chemical analysis the tissues were minced and subjected to the following analyses.

Iron. Determination of "the easily split off" iron in bruised tissue, resulting from the degradation of hemoglobin present in the bruise, was carried out with 20 grams of samples. Samples were homogenized in a Waring 1/ blendor with 150 ml. of 0.4 percent HCl for 3-5 minutes. The slurry was transferred to a beaker; the Waring blendor and the cap were each washed three times with 10 ml. of 0.4 percent HCl; and the contents were added to the slurry. After incubation for 24 hours at 37° C. (Barkan and Walker, 1), the protein was

1/ Mention of trade names in this report does not imply approval of the Department of Agriculture to the exclusion of similar products.

precipitated by the addition of 20 percent trichloracetic acid 1:2. The iron in the protein-free filtrate was determined photometrically as the ferrous compound of o-phenanthroline (Fortune and Mellon, 2).

Pigment extraction with saline. Ten grams of minced tissues were homogenized in a Waring blendor with 60 ml. of buffered saline 2/. The slurry was transferred to a centrifuge bottle. Residual tissue was removed from the blendor by washing with 10 ml. of saline. This, in turn, was added to the original slurry. After centrifugation for 30 minutes at 30,000 x g, the supernatant fluid was filtered and the sediments were resuspended with 10 ml. of saline, centrifuged and filtered. This procedure was repeated and the filtrates were combined and mixed and the volume recorded.

Pigment extraction with chloroform. The sediments remaining after the saline extraction were mixed in a Waring blendor for 2 to 3 minutes with 50 ml. of chloroform and then quantitatively transferred to a centrifuge bottle. After centrifugation at 30,000 x g for 15 minutes, the supernatant fluid was filtered and the sediments were resuspended with 10 ml. of chloroform, re-centrifuged and filtered. This procedure was repeated and the filtrates were combined, thoroughly mixed and the volume recorded as before. The concentration of bilirubin was determined spectrophotometrically at 450 m u according to a standard curve constructed using known concentrations of bilirubin in chloroform.

Hemoglobin and bilirubin. For qualitative studies of these compounds and other pigments in the extracts, the American Optical Company Rapid Scanning Spectrophotometer, Model 1 A, was used and the general procedure reported by

2/ Saline buffered to pH 7.4 and containing 1.0 percent normal human serum albumin. This solution is referred to as saline.

Shinowara (3) was employed. Sometimes the scanning was made on various tissue sections using the reflection attachment of the instrument.

The concentrations of the hemoglobin and the bilirubin in the saline extracts of the control and of the bruised tissues were determined with a Beckman D. U. spectrophotometer and a 1 cm. cell. Optical densities were measured at the following wave lengths: 450, 560 and 575 mu, and the concentrations of both hemoglobin and bilirubin in the mixture were determined as reported by Shinowara (3). No correction for carotene and other lipochrome was made in these studies during the determination of these pigments. Preparations of standard hemoglobin and stable bilirubin were made as described by Shinowara (3).

Non-protein nitrogen. The non-protein nitrogen of the bruised and normal tissues was determined from saline extracts prepared for pigment analysis. Two grams of trichloroacetic acid crystals were added to 10 ml. saline extract and the mixture was thoroughly shaken to precipitate the protein and then filtered. The protein on the filter paper was washed twice, with 1 ml. distilled water. The total protein-free filtrates were combined, the volume recorded and the nitrogen determined on 1 ml. filtrate by a micro-Kjeldahl method using sulfuric acid and hydrogen peroxide for digestion. The ammonia formed was determined by direct nesslerization of the digestion mixture using a slight modification of the procedure suggested by Miller and Miller (4).

Application of hemoglobin and bilirubin analyses. Barkan and Walker (1) established that there is an increase in the iron content of plasma from whole blood, containing anticoagulant, following incubation at body temperature.

Best and Taylor (5) reported that in blood the non-protein nitrogen concentration rises under conditions which are associated with excessive tissue catabolism. Selye and Dosne (6) found that blood which passed through a damaged area contained a high concentration of hemoglobin and non-protein nitrogen.

Since bruises are characterized by hemorrhage, the fate of the hemoglobin in a bruise was investigated by measuring the iron content (the easily split iron fraction resulting from the hemoglobin degradation). The non-protein nitrogen of bruised tissue during the progressive process of healing was also investigated.

Bruised and control tissues were excised and analyzed at different stages of healing for iron and non-protein nitrogen. This experiment was repeated several times.

The iron and non-protein nitrogen content of a bruised tissue varied considerably from one bruise to another, as might be anticipated, depending on the extent of the damaged tissue. In most of these experiments the iron content was found to increase after the infliction of the bruise, followed by a trend toward the value of the normal tissues (0.25 mg/100 grams of tissue sample) within 7 to 10 days, depending on the extent of the bruise. This increase of iron (E.S.I.) content during healing indicated that the hemoglobin present in a bruise was catabolized with the concurrent removal of its iron content.

The non-protein nitrogen in the bruised tissue was found to decrease from the control level of 650 mg percent to 140 mg percent in 5 days, but

rose abruptly to the control value in 9 days. No explanation was found for the drop of the non-protein nitrogen in the early stages of a bruise, but the higher concentration of this non-protein nitrogen after 4 to 5 days might have been due to the excessive tissue catabolism and the degradation of the globin portion of the hemoglobin molecule. The increase of iron coincided with the decrease of non-protein nitrogen up to the fifth day, after which time the reverse was observed.

Spectrophotometric studies on saline extract of bruised tissue during healing indicated that the extra-stromal hemoglobin concentration increased immediately after the bruise occurred, followed by a decrease to reach the level of normal tissue upon complete healing. The concentration of the yellow pigments, representing the total of that found in both the saline and chloroform extracts, was then determined spectrophotometrically with C.P. bilirubin (Eastman) as a standard. The results showed that the yellow pigments in normal tissue ranged from 0.32 to 0.86 mg/100g., while that of the bruised tissues increased to a level of 4.3 within 4 days of injury, followed by a rapid decrease to reach the normal tissue level.

Total bilirubin was then determined chemically by the Van den Bergh reaction on the saline and chloroform extracts of control and bruised tissue. The results indicated that the control tissue extract did not contain bilirubin, per se, while its concentration in the bruised tissue extracts rose to 3.8 mg/100g. tissue within 4 days after injury and then decreased to a level of 0.20 mg/100g. by the ninth day. Therefore, the increase in the total yellow pigments due to injury was attributed to bilirubin. This was confirmed by isolating the pigment

as the barium salt: The free pigment had a maximum absorption at 450 mu identical to that of C.P. bilirubin. The yellow pigments in the normal (control) tissue must have been substances other than bilirubin, such as lipochromes, riboflavin and hemoglobin, all of which absorb light at 450 mu.

Color Test for Determining Age of Bruises

On the basis of the experimental observations delineated above, a color test was devised which makes use of the important factors of bilirubin formation in bruises.

Bilirubin in bruises. Several animals which had one bruise per animal were used in this experiment under controlled laboratory conditions. At various intervals during the healing process, bruised and control tissues were excised, scored and tested for the presence of bilirubin. The bilirubin seemed to be bound to the protein molecules of the tissue confirming the results reported by Stenhager and Rideal (1936). The tissues were immersed in Fouche's reagent for 10-20 minutes at room temperature and then examined.

The results of these experiments are recorded in Table 2 and indicated the absence of bilirubin in bruised tissue of less than 50-60 hours of age and in control (non-bruised) tissue. The color reaction of a bruised tissue was a diffused light blue at the age of 60-72 hours and diffused dark green when measured on the fourth and fifth days after infliction of the bruise. This color reaction was most discernible at the periphery of the bruise and decreased in intensity towards the center, analogous to the yellow-green (orange) color usually observed on the skin over a bruised tissue. This seemed to be

Table 2. - The color reaction of bruised tissues at various intervals during the healing process in cattle

Age of the bruise	Tissue color after 10-20 minutes incubation in the Fouche reagent at room temperature
Normal tissue	The tissue developed no color.
Zero-60 hours (fresh bruise) (under 60 hours old)	The color of the bruised tissue was pink in the first few minutes and gradually turned brown. No blue color was detected.
60-72 hours (intermediate bruise)	The tissue developed a diffused very light blue color along with a dark brown color at 60 hours and blue at 72 hours. This blue color could be clearly observed in the fat tissue.
3-5 days (slightly old bruise)	A diffused dark green color, especially at the periphery, along with a brown color (in the center) of the bruised tissues were detected.
5-8 days (old bruise)	This type of bruise developed slight or no blue color, but upon longer contact with the reagent[1], dark green crystals were found imbedded in the bruised area of either the lean or the fat tissue.

[1]This color reaction was detected when the tissue was allowed to remain 2-6 hours in the Fouche's reagent.

due to the hypermetabolic activities of the undamaged tissues at the periphery of the bruised area.

After 5 and up to 8 days this bile pigment was found to localize in high concentration in various areas of the bruised tissue, most probably in macrophages. Therefore, the diffused color reaction was very light blue or absent and instead dark green areas or crystals (2-3 mm in diameter) were found when the tissue was allowed to remain longer (2-6 hours) in the Fouche's reagent, thus permitting more time for the reagent to diffuse and reach the high local concentration of bilirubin imbedded in the tissues. This bile pigment may undergo autoxidation when the atmospheric oxygen is sufficient and this test should be made directly upon slaughtering the animals.

Experiments on the hide of the bruised animals using this color test indicated its usefulness with the hide alone or in conjunction with bruised tissue for the determination of the age of the bruise. The same types of color reactions were obtained on the hide of the animals only in and around the bruised areas, suggesting the possibility of biopsy experiments to determine the age of a bruised tissue in the living animal.

Specificity of the color reaction. Studies to investigate the specificity of this color test were conducted using biliverdin and other pigments which were established to be present in bruised tissue, such as hemoglobin, alkaline hematin, and biliverdin (Hamdy et al., 1956). Various concentrations of these pigments were adsorbed on a layer of talc in a Buchner funnel and tested for color development using Fouche's reagent (Naumann, 1936).

Bilirubin was the only compound among the pigments tested which produced

the blue and green color with Fouche's reagent. The change of color from light blue to dark green depended on concentration of the adsorbed bilirubin; high concentration developed a dark green color. Alkaline hematin produced a pink color which immediately turned to brown while hemoglobin produced brown color. Biliverdin displayed no color with Fouche reagent.

Sensitivity of test to bilirubin. Naumann (1936) reported that the limit of detectability of bilirubin dissolved in weak NaOH, dilute alcohol and urine, freed from preformed bilirubin, were 0.006, 0.008 and 0.009 parts per million, respectively. Since bilirubin appeared to be bound to the protein in the tissue, it was decided to investigate the limit of detectability of bilirubin albuminate solution (pH 7.4). The results revealed that the limit of detectability of bilirubin albuminate absorbed on a layer of talc was 0.01-0.008 p.p.m.

Further experiments were conducted using cattle tissues (lean and fat) for the adsorption of bilirubin albuminate. The tissues were cut to rectangular pieces 2 x 0.5 x 0.12 inches. One ml. containing various concentrations of bilirubin albuminate ranging from 1000 to 0.00001 p.p.m. was placed on each tissue sample. After 4 hours at room temperature in open atmosphere, or under vacuum for 2 hours, the adsorbed bilirubin was detected by adding 2 ml. of Fouche's reagent to the surface of the tissue. The results of these experiments showed that the limit of detectability of bilirubin albuminate adsorbed to the tissues was 50 to 100 parts per million.

Detection of the color was easier in fat tissues than in lean tissues and adsorption of 50-100 p.p.m. bilirubin albuminate produced a light blue

color which changed in intensity to reach a dark green color when the tissue adsorbed higher concentrations of bilirubin.

Effect of different tissues and species of animals. Tissues from various muscles of test animals were tested with one ml. of bilirubin albuminate containing 100 p.p.m. of bilirubin albuminate. The results showed that the color test was not affected by location of muscle sample or animal species.

Conductivity as a Measure of the Age of Bruised Tissue

During studies on bruises, iron, hemoglobin, bilirubin, non-protein nitrogen and accumulation of fluid were found to be correlated with the age of a bruised tissue (Hamdy et al., 1956). For example, hemoglobin and the accumulation of fluids were found to be higher in a bruised tissue than in a normal tissue, especially in the early stages of the bruise.

The accumulation of the fluid in a bruised tissue with its increased concentration of electrolytes was considered to be another aspect of the problem. It was recently reported by Merezhyhskii and Charkasava (1954) that the concentration of sodium increased in a bruised tissue, and then decreased to normal upon healing. They also stated that under conditions of trauma cellular barriers were destroyed with respect to Na ions, resulting in an increased diffusion of sodium ions into the cells of the traumatized tissues and in a greater accumulation of Na^+ in the intercellular fluid.

Thus, the conductivity of normal and bruised tissue in cattle which had suffered one bruise was measured 24 hours after the injury. The average resistances offered by the bruised tissues and by the control (non-bruised tissue) were obtained immediately after removing the hide. The control tissues

were excised again from areas located symmetrically from the bruised tissue. The resistance offered by the tissues was measured and conductivities of the tissues were calculated by dividing the cell constant by the average resistance offered by the tissue. This experiment was repeated several times and the mean conductivity value for a 24-hour-old bruise was found to be (4.6×10^{-2} ohm^{-1} cm^{-1}) $\pm$ 0.4 while the control sample was (2.07×10^{-2} ohm^{-1} cm^{-1}) $\pm$ 0.1 indicating a significant difference in conductivity between bruised and normal tissue.

To investigate the relationship between conductivity of bruised tissue and its age during the healing process, tissue from cattle having similar bruises but at different stages of healing was examined. Resistance measurements were made from which conductivity values were calculated (Table 3). The conductivity of bruised tissue was affected by the age of the bruise, increasing during the early stages of healing to reach a maximum after 40 hours, and then decreasing to the normal value of the control upon complete healing. This suggested that the concentration of electrolytes increased in the first stages of healing and then subsequently descended to the normal value as healing is completed. Some variations were observed between the conductivity of different bruised tissues of the same age. This was found to be due to many factors, such as the size and the extent of the bruise and on the quantity of fat present in the tissue. When the conductivity of the bruised tissue and the control were plotted against the age of the bruise (Figure 1), it was possible to approximate the age of an unknown bruise.

Significance of Various Factors on Bruising and Wound Healing

During the investigations there were indications that the time required

for gross changes to occur in bruised tissue was variable and that extent of damage varied with different animals. In general, however, the sequence of visible changes was always consistent regardless of species, character of the bruise, or bruise location. Because of these findings, studies were undertaken to examine some of the factors which might affect bruising of both large and small animals.

Influence of previous bruising on rate of healing. A number of test animals were divided into several groups. One group of animals had only one bruise. A second group had suffered two bruises, the second bruise having occurred 3 days after the first bruise. A third group of animals had suffered three bruises, the second 3 days after the first and the third bruise 2 days after the occurrence of the second. All the animals were examined grossly prior to slaughter and the tissues examined by the Fouche's reagent for bilirubin (Hamdy, et al., 1956) immediately after slaughter. The results recorded in Table 4 indicate that the time required for complete healing as determined by gross observation and by the detection of bilirubin was shorter for each subsequent bruise. The second bruise, in the case of animals with two bruises, healed in an average of 6.6 days as compared to 7.9 days for those animals that had only a single bruise. The last bruise of those animals which had three injuries healed in an average time of 5.9 days; that is, faster than either the first or second bruises sustained.

Similarly, bilirubin was detected earlier in the tissues following each subsequent bruise (70 hours, single bruise; 56 hours, second bruise; 46 hours, third bruise).

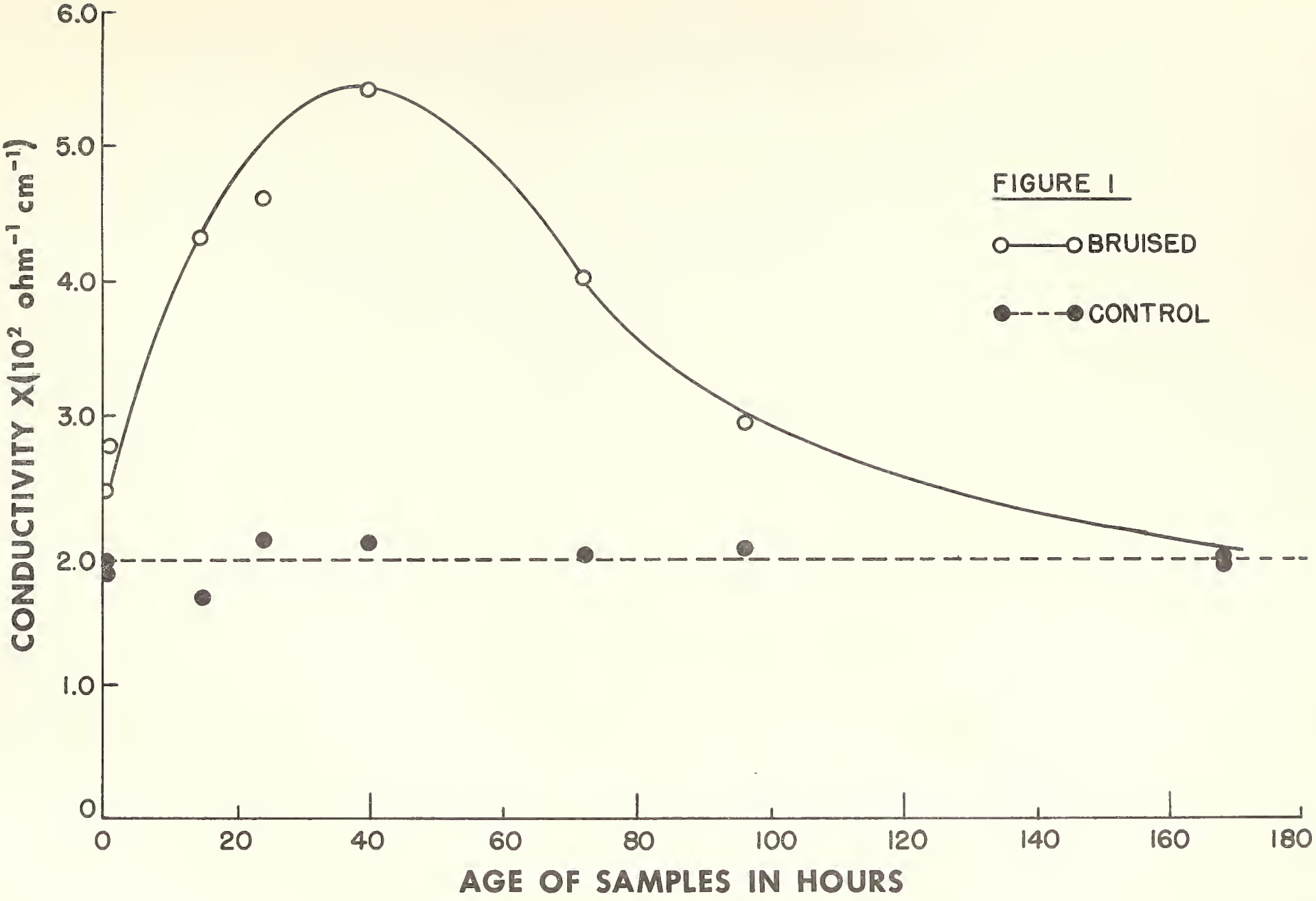
6.0
5.0
4.0
3.0
2.0
1.0
0
CONDUCTIVITY X(10² ohm⁻¹ cm⁻¹)
0
20
40
60
80
100
120
140
160
180
AGE OF SAMPLES IN HOURS
FIGURE 1
BRUISED
CONTROL

Table 3. - The relation between the conductivity and the age of a bruised tissue in cattle

Age of sample	No. of samples	Conductivity x 10^{-2} (ohm-1 cm-1)			
		Bruised	St. Dev.	Control 1/	St. Dev.
Just before killing	5	2.45	0.3	1.80	0.2
15 minutes	4	2.75	0.5	1.86	0.2
15 hours	6	4.30	0.6	1.65	0.3
24 hours	8	4.60	0.4	2.07	0.1
40 hours	7	5.40	0.3	2.05	0.1
3 days	6	4.00	0.3	1.95	0.2
4 days	6	2.95	0.3	2.00	0.1
7 days	5	1.95	0.2	1.90	0.2

1/ Control from a symmetrically located area as the bruised tissue sample.

Table 4. - Healing time and bilirubin detection in test animals having multiple bruises

No. of animals	Healing time (days)		Bilirubin detection (hours)	
	Mean	St. Dev.	Mean	St. Dev.
		First Bruise		
40	7.9	0.3	70	1.4
		Second Bruise		
40	6.6	0.2	56	1.5
		Third Bruise		
40	5.9	0.1	46	1.8

Table 5. - Passive transfer of healing factor

No. of bruised animals tested	Source of blood transfused (20.0 ml.)	Healing time (days)		Bilirubin detection (hours)	
		Mean	St. Dev.	Mean	St. Dev.
5	Bruised animals	6.1	0.3	43	1.6
14	No treatment (control)	7.9	0.3	70	1.4
4	Normal animals	7.8	0.2	72	1.4
3	No treatment other than injection of 3.0 ml. of anti-coagulant 1/	8.0	0.3	71	1.5

1/ A.C.D. solution. Each 100 cc. contains: 2.45 gm dextrose, 2.20 gm sodium citrate and 0.80 gm citric acid.

Attempts to passively transfer the healing factor. In view of these results an experiment was conducted to investigate the possible transferability of the "healing factor" or factors in the blood of a bruised animal.

A rabbit suffered four bruises at weekly intervals. Five days following the last bruising, 20.0 ml. of blood were withdrawn by cardiac puncture into 3.0 ml. of anticoagulant 3/. This blood was injected intravenously into a normal animal which suffered a bruise 24 hours after the transfusion. Animals treated in the following manner served as controls:

(1) Bruised animal which had not received a blood transfusion.

(2) Bruised animal previously injected with a 3.0 ml. of the anticoagulant.

(3) Bruised animal previously injected intravenously with 20.0 ml. of normal blood from a non-bruised animal.

Several other groups of animals were treated similarly. At various intervals the test groups were slaughtered, and the bruised tissues were examined grossly and tested for bilirubin as before.

The results recorded in Table 5 demonstrate the accelerated healing of animals transfused with blood from bruised animals. Complete healing of bruised tissues in the transfused animals, as determined grossly, occurred in an average of 6.1 days as compared to 7.9 days for the control animals. The presence of bilirubin also was detected earlier in the test animals (43 hours as compared with 70 hours).

Effect of Vitamin C Content with Diet. Vitamin C has been implicated by many workers to affect capillary fragility and rate of wound healing so a

3/ A.C.D. solution. Each 100 cc. contains 2.45 gm dextrose, 2.20 gm sodium citrate and 0.80 gm citric acid.

brief study of this was undertaken. A group of healthy animals of the same age were used in this experiment. They were divided into two subgroups, A and B. Subgroup A were fed on a diet which contained inherent traces of Vitamin C. Subgroup B were fed on the same diet except it was fortified with Vitamin C to contain 8 m. gm. per 100 gm. of feed. The animals in both subgroups suffered similar bruises on the gluteus muscles. The damaged tissues were examined daily and before and after slaughtering, which was made after 3, 5, and 7 days from the time a bruise was suffered. The results of this experiment showed that the animals which were fed on Vitamin C fortified diet had a tendency to heal faster. The size of the damaged tissues of subgroup B were smaller than those of subgroup A, indicating the important role of Vitamin C on the healing process.

It may be that this excess Vitamin C is taken up by the tissues during the healing process or that this vitamin can act as an activator in the degradation of the hemoglobin molecule to bilirubin. Bilirubin was detected in the damaged tissues 55-50 hours after bruising in subgroup B animals as compared to 60-72 hours in subgroup A, which lends support to the approach that Vitamin C can act as an activator in the degradation of the hemoglobin.

Effect of Streptokinase-Streptodoranase varidase. Studies were directed using an enzyme preparation, namely "Streptokinase-Streptodoranase varidase". This combination of enzymes are produced by a strain of Streptoccus by Lederle Laboratories. It was reported that the Streptokinase acts directly upon a substrate of fibrin or fibrinogen by activating a fibrinolytic enzyme, while the Streptodoranase acts upon a

substrate of neucleoprotein of the dead cells and has no effect on living cells. (Sherry, S. and Goeller, J. P. J. Clin. Investigation 29, 1588, 1950).

The effect of the inoculation of this preparation on bruised tissues was studied with the aim of using it as a means to accelerate the healing processes of these tissues, either directly or indirectly.

Various experiments were conducted and the following is a summary of these experiments.

1. Maximum dose. Subcutaneous and intramuscular injections of various concentrations ranging from 2,500 to 30,000 units Streptokinase were made in animals in order to establish the maximum dose which causes no visible reaction on the skin of the animal. The results of this experiment indicated that injection of 20,000 units or more showed inflammatory reactions on the skin of the rabbits weighing 6-8 lbs.
2. Effect of the Streptokinase, etc., on the healing of bruised tissues in cattle and rabbits. A cow suffered bruises on four different areas. One bruise was injected intramuscularly with 15,000 units Streptokinase, the second received 30,000 units, the third 45,000 units, and the fourth a control. Forty-one hours after bruising, the bruised tissues were examined morphologically. Color test and conductivity measurements were performed on the bruised tissues. The results of this experiment showed that the inoculation of the enzymes produced a very marked effect on the four bruised areas. Almost all the four bruises healed as observed morphologically, while their

conductivity values were about the same as a non-bruised tissue. This experiment was repeated on a number of cattle and rabbits with conflicting results.

Some bruised animals responded to the treatment with the enzyme and rapid acceleration of healing was observed as described, while other animals did not respond. Histological study of some of these tissues only confirmed the gross observations and no new leads were uncovered. It is possible that these animals which did not respond to the treatment with the enzymes may have had a recent streptococcal infection and hence might have had a high titer of antibodies specific against the injected enzymes.

Effect of trypsin. Experiments were conducted using bruised cattle. Intramuscular injections of 1 ml. "Parenzyme," containing 1 mg. crystalline trypsin, were performed daily on the experimental animals. At various time intervals during the healing process, the bruised tissues were examined. Bruised animals receiving no "Parenzyme" served as controls and were also used for comparison. The results obtained from these experiments indicated that intramuscular injection of the "Parenzyme" seemed to be of value in the treatment of bruises. Some animals responded to the treatment as evidenced by the reduction in the size of the bruised tissue and acceleration of the rate of healing of the damaged tissues.

Rate of healing in different animal species. The damaged areas of bruised cattle, hogs, and sheep were examined grossly before and after slaughter. Tissue samples also were tested for bilirubin formation. In

general, the rate at which the gross changes associated with bruising and healing occurred was approximately the same, regardless of the species being studied. The presence of bilirubin in the damaged tissues of the various animals was first observed between 60 and 72 hours from the time of bruising. It should be stated, however, that some differences were noted between individuals of the same species.

Influence of age on rate of healing. Eight young animals of varying ages each suffered a single bruise over the gluteus muscle. Tissues from two animals in each age group were analyzed after 55 hours, 70 hours, 3 days and 7 days from the time of bruising, with the bruised tissues at the latter two intervals being excised and weighed. Bilirubin was detected by the Fouche's reagent in the tissue 55 and 70 hours old. Although the number of animals used in this experiment was not large, it can be stated that the bruises on the younger animals appeared to heal significantly more rapidly than did bruises on older animals. Also the quantity of damaged tissue in the older animals was observed to be greater than that found in the younger animals. Bilirubin was detected at 55 hours in the younger but not in the older animals, indicating acceleration of hemoglobin degradation (a measure of healing).

Relationship of force to extent of bruising. A number of cattle suffered bruises on the biceps muscle. These injuries were sustained in different ways, i.e., the bruises were brought on by contact with objects of varying size and contacts took place with varying force. At various times during the healing process, the bruised tissues were examined and compared.

The results of these tests indicated that the extent and the severity of the damaged tissues were affected directly by both the mass of the object contacted and force with which the contact was made. A large mass and a negligible force produced minimal or no bruising, while a small mass struck with severe force resulted in a small, discrete but severe type of bruise. When both the mass of the contacted object and force of contact are increasingly greater, the area and depth of the bruised tissue also are increased. This in turn leads to a prolongation of the time necessary for complete healing when compared to the time required for the superficial type of bruise. Regardless of the force with which contact was established, the sequence of visible and chemical changes associated with healing were the same.

Location of the bruise. Several cows suffered similar bruise injuries on various parts of their bodies. At the time the bruise occurred, bodily contact with the object causing the bruise was made with approximately the same force in the case of each animal. The bruised tissues were examined grossly before and after slaughtering.

The bruises over the gluteus muscles, the triceps, the biceps and the trapezius muscles were deeper than were those over the lubo-dorsal fascia and the serratus muscles. Bruises over the latter muscles tended to spread laterally. More tissue damage with accompanying hemorrhagic edema, extending into the intermuscular tissues, was observed when the bruises were close to the tuber coxae, tuber ischii, the scapula and the patella.

Influence of blood pressure on bruising. Certain cattle suffered bruises going to slaughter. After stunning, some of the animals suffered other bruises when they fell. After slaughter and bleeding, an attempt was made to bruise the carcass of the dead animal. The bruised tissues were examined by the usual procedures.

The results bore out the contention that animals may bruise before and after stunning, and before, but not after, bleeding, i.e., when the blood pressure approaches zero.

Relationship of Transportation Factors to Bruises

In order to provide some indication of the possible answers to the questions surrounding bruises in transportation, test lots of cattle were transported under controlled conditions from Wooster to Columbus (about 100 miles). Records were kept of the loading conditions and arrangements, possible bruise-causing incidents en route, and unloading and holding conditions. All cattle were identified by number and slaughtered at various times with the bruises catalogued and then given laboratory examinations.

Test I - This test involved seven head of 1000 pound steers which had been stanchion fed for several months and which were deemed to be free of bruise or injury before loading. The animals were loaded with such care that it can be said no bruising occurred during loading. The truck was well bedded with fresh, clean straw over sand.

Four of the cattle were placed side by side in the forward end of the truck alternately facing to the front or rear. A divider was placed tightly against them to restrict movement in transit. The remaining three head were

given the rest of the truck (about half of the total area). This allowed these latter animals to shift with the movement of the vehicle.

In addition to bumps and jolts which were sustained over a chuck-hole detour, the following potential bruise-causing incidents en route were noted:

19 sudden or rough stops.
8 jerky starts.
4 quick turns to the left.
6 quick turns to the right.
2 stops and starts on a steep grade.
5 rough or jerky gear changes.

The three animals in the rear of the truck and one of the animals in the forward end were slaughtered the following day. The steer from the forward end showed severe bruising around the pin bones where starting and stopping allowed this animal to bump the divider. The steer in the extreme rear end of the truck was slightly and superficially bruised on the side exposed to an adjacent animal. The steer positioned next to the divider in the rear end of the truck showed severe bruising along the left side where constant contact with the divider was made. No bruises were evident on the third steer which was positioned in the middle of the rear half of the vehicle between two animals.

Test II - The same general conditions were prevalent as in Test I except for the loading arrangement. In this test the seven cattle were allowed to move about in the truck at will. The log of bruise-causing incidents en route was as follows:

15 sudden stops.
3 jerky starts.
5 quick turns to the left.
3 quick turns to the right.
2 rough or jerky gear changes.
20 abrupt braking occasions.

Two of the steers were thrown down in the truck during the trip when the truck brakes were applied abruptly.

Following slaughter, careful observation disclosed no bruises except around hocks and shanks. These bruises were deemed to have occurred when the cattle went down on the slick surfaces of holding pens and scales.

The small number of animals involved in these two tests permits only these very general observations to be made:

(1) Individual animals differ in their susceptibility to bruising.

(2) Loading arrangement may be more of a factor in bruising than lack of consideration by the truck driver.

Summary and Conclusions

The first approach from the standpoint of biochemistry and pathology was to obtain animals which had suffered injury at various times and examine the tissue to observe morphological and chemical changes. The more obvious factors which were changing in the damaged tissue could be arranged in certain sequences. For example, the normal red cell of the blood finds itself in a bruise outside the circulatory system. When the wound is completely healed, all of these red cells are replaced by repaired tissue. It was possible to follow the changes in these red cells microscopically. Chemically one of the most obvious changes in bruised tissue is that of color. Everyone is familiar with the black and blue spot of a new bruise which changes to green and yellow and then disappears. Microscopic examination can follow the changes in the red cell and the biochemist can follow the changes in the red pigment, hemoglobin, as it first appears in the damaged tissue and as it is degraded through the

purple or blue-black, the yellow and green stages and finally its disappearance.

In neither instance of chemical changes or histological changes was there much variation in the actual sequence of events. For example, event A was followed by event B, by event C, and the like. The confounding problem was that the time between event A and event B, or the time between event B and event C, and the like, was not constant between species or within a species. That is to say some bruises would be completely healed in 15 days in some animals and 7 or 8 days in others. What, then, was controlling these processes?

To get some answers on this problem, animals were used which had suffered wounds on different days ante-mortem. It was observed that chemical and microscopic examination of these bruised tissues did not, in fact, produce results that precisely followed the known times of the various wounds. That is to say that if a steer was wounded, the first wound could affect the rate of healing of the third or even the third might affect the rate of healing of the first. So not only was the healing rate different in different animals, the healing rate was different within a single animal. The biologist might call this a "refractory" process, or a process which is under humoral or chemical control. Humoral control means that a substance is produced in one part of the body, say the damaged tissue, which controls certain processes in other parts of the body, as perhaps in another wound.

To pin this down in still another way, if wound healing or the changes in bruised tissue are in fact under this type of control, then it should be

possible to take the blood of a wounded animal after the stimulus for healing is produced, transfer it to a normal animal and in this so-called normal animal the rate of wound healing should be faster than a comparable animal which had not been transfused. This is precisely what happened. In other words, it was possible to transfer from one animal to another animal substances which would alter the healing rates.

These observations pointed up a corollary possibility. Even though up to this time no biochemical process was uncovered which was independent of time, there appeared to be the possibility that animals could be treated in some way to hasten the repair of bruised or damaged tissue. This proposition was studied. The first problem was what were these factors which could influence the rate of healing? If these were known, then they might be administered to animals in such a manner as to decrease carcass meat losses. A decrease in loss was the ultimate goal. Toward this end, then, a number of exploratory experiments were conducted.

The reasoning behind this approach follows. When wounded tissue is repaired, the damaged cells or the improperly placed cells, such as red cells in muscle tissue, must be liquidated. The damaged tissue is protein. This proteinaceous tissue must be disintegrated or digested before it can be replaced by new tissue. The biochemical way in which this can take place is through enzymes which can disintegrate the damaged tissue. Could it be then that the humoral substances controlling wound healing were, in fact, certain enzymes whose function was the dissolving of damaged tissue?

Experiments were undertaken to test this hypothesis. Trypsin and some other proteolytic enzymes were injected into animals to determine if they could influence the rates of healing. Both small animals and large meat animals were studied to determine if injections of certain enzymes would alter healing rates. This appeared to be the case. Hence, the point was established that the biochemical processes involved in wound healing could in fact be controlled by external stimuli as well as by stimuli within the animal itself. Although this was a very gratifying and interesting fundamental finding from the scientific point of view, it pointed up the remote possibility of finding a simple test which might be applied to damaged tissue which would unequivocally tell the age ante-mortem at which the wound was inflicted.

While this work involving large animal experimentations fell short of the original objective of ascertaining precise intervals between time of bruising and time of examining bruised tissue, useful information was obtained--information which if used properly could lead to substantial reduction in bruise losses.

The necessity of procuring information on all time intervals of practical interest required studies designed to establish whether or not animals might suffer bruise injury in the usual sense after stunning as well as before. It was found that as long as approximately normal blood pressure was maintained in an animal, bruising could result. This was established by testing tissues of animals that suffered bruises after having been stunned in the usual slaughtering process. Animals did bruise until after normal

blood pressure dropped as result of sticking and bleeding--the usual pattern in commercial slaughter.

What this means is that until animals are shackled and bled, bruise damage is possible. Consequently, it appears that close scrutiny is in order in handling animals in the holding pens, runways, and slaughter facilities of packing houses.

All persons who have visited packing houses are aware of a great degree of variability in the emotional state of animals prior to slaughter. Evidence obtained tended to support the view that highly excited animals appeared to bruise more easily than relaxed ones.

In the trucking experiments which were conducted, and also in other experiments, it was quite apparent that fleshier animals bruised less readily than thin ones. Also bony parts of the animals bruised more easily than the fleshy parts.

It is particularly worthy of note that in two trucking experiments with very docile animals which had been stanchion fed for months prior to slaughter that the manner of loading as well as roughness of ride were important. Animals should not be tightly confined, nor allowed sufficient room in trucks to be able to move about. The least bruising was apparent in animals confined but allowed a little room. Such loading tends to diminish shock of bumps and sudden starts and stops. In these series of tests no cattle were transported by rail, but in the trucking studies most of the wounds seemed to be rather superficial and only a very few were deep bruises.

In studying random packing house bruises it was not possible to pinpoint time of bruises to better than a ± 30 percent, but the tests which were applied could usually indicate that at least some of the bruises were inflicted during transporting to the packing house and handling in the yards and in handling immediately prior to slaughter. Although many of these bruises appeared to be superficial, it was not possible to state that deep and large bruises occurred farther back in the animal's history and the superficial ones were the ones closer to the time of slaughter.

The bilirubin color and other chemical tests developed in this project, as well as the conductivity and other physical tests developed, can place the age of bruise within 30 to 50 percent accuracy. The reasons for this already have been explained. Even though such accuracy is insufficient to unequivocally state the time of injury, these tests are sufficiently accurate for statistical type studies wherein damage patterns can be established. That is to say, if it were desired to study long-haul rail transportation against long-haul truck transportation, or long hauls versus short hauls, or any other variations of animal management immediately prior to slaughter, it would be possible to study bruises at slaughter in such a way as to determine which procedures produce the greatest losses. By this it is meant that the tests can be used for determining bruises which might occur within 24 to 36 hours as compared to bruises which might have occurred 4 to 5 days earlier. It might be possible to differentiate bruises in packing houses against those suffered on the farm, providing there is a long enough

time interval between the farm and slaughter. Even though one can recommend these tests for statistical treatment of large groups of livestock handled in various ways, the tests cannot be recommended for use as establishing individual liabilities several days before slaughter. These tests in conjunction with other known information, however, might be supplementary in providing suitable evidence concerning responsibility for losses.

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